Effects of diet and exposure to hindlimb suspension on estrous cycling in Sprague-Dawley rats

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Tou, Janet C. L., Richard E. Grindeland, and Charles E. Wade. Effects of diet and exposure to hindlimb suspension on estrous cycling in Sprague-Dawley rats. Am J Physiol Endocrinol Metab 286: E425–E433, 2004. First published November 18, 2003; 10.1152/ajpendo.00287.2003.—Various factors can disrupt the female reproductive cycle resulting in subfertility. The primary objective of this study was to determine whether physiological changes associated with exposure to hypogravity disrupt reproductive cycles. The hindlimb suspension (HLS) model was used to simulate the major physiological effects of hypogravity in female Sprague-Dawley rats. Also, to determine whether diet may influence reproductive results, rats were fed purified American Institute of Nutrition (AIN)-93G or chow diet. Rats (n = 9–11/group) subjected to HLS had lengthened estrous cycles due to prolonged diestrus, indicating hypoestrogenism. Interestingly, HLS rats fed AIN-93G but not chow diet had significantly reduced time spent in estrus and decreased plasma estradiol. Attenuation of hypoestrogenism in the chow-fed rats suggested that diet provided an exogenous source of estrogen. The mechanism involved in the disruption of estrous cycling remains to be determined. HLS increased urinary corticosterone (CORT) levels during the initial 4 days of HLS, suggesting that physiological responses to acute stress may be a potential mechanism in the disruption of estrous cycles. Higher basal urinary CORT was observed in rats fed chow vs. AIN-93G diet. HLS resulted in increased urinary CORT. However, two-way ANOVA indicated a significant HLS effect (P < 0.001) but no effect of HLS × diet effect on urinary CORT levels, suggesting that estrogenic activity associated with the chow diet did not enhance the stress response. The results of this study indicate that HLS, diet, and the combination of HLS and diet influence estrous cycling. This has important implications for future reproductive success in the hypogravity environment of space.

hypogravity; estrogen; reproduction; stress; phytoestrogen

REPRODUCTIVE DISORDERS AND HAZARDS to reproductive health have become prominent public health issues (9). Determining the role of environment on reproduction provides a better understanding of causes of reproductive dysfunction as well as insight regarding the impact of novel or extreme environments on fertility.

In space flight, reduced testosterone (T) has been observed in male astronauts (33) as well as in male rats (1). In view of the sex hormone changes in males following space flight, it is presumed that space flight will similarly affect female sex hormones. The number of women astronauts is increasing, and many may not have started or completed their families at the time of space flight, yet little is known about the effect of space flight on female reproduction. Serova and Denisova (32) reported that rats mated during space flight but no births resulted. No human data exist because women astronauts suppress their menstrual cycles during space flight (17). Clearly, more studies are needed to answer basic questions about female reproductive function in space.

In the space environment, hypogravity produces physiological changes, such as musculoskeletal disuse atrophy, headward fluid shift, and adaptation stress, which may affect reproduction. This has important implications because the time spent under hypogravity conditions is expected to increase as the duration of space flight missions lengthens. Because of the cost and rare opportunity to conduct space flight studies, ground-based simulation models of space flight have been developed. To study the effect of hypogravity, humans are subjected to 6° head-down-tilt bed rest. Women subjected to 17-day 6° head-down-tilt bed rest showed no changes in menstrual cycle length (31). However, the 17-day duration of the study was too short to draw definitive conclusions. In another study, Rock and Fortney (29) reported that women exposed to bed rest exhibited luteal phase deficiency.

In animal studies, the Morey-Holton hindlimb suspension (HLS) model has been used successfully to simulate the major physiological effects produced by exposure to hypogravity (21, 22). The HLS model involves suspending rats by the tail base to produce a 30° head-down position, which prevents the hindlimb from bearing any weight, as well as the headward fluid shifts experienced in the hypogravity environment of space. Congruent with the space flight results, HLS of male rats resulted in decreased testis mass and reduced plasma T (1). To our knowledge, no studies have investigated the effects of HLS on reproductive function in nonpregnant female rats.

We also examined diet because the current space flight diet consists of a semipurified formulation, whereas ground-based studies such as HLS typically use a standard chow diet (37). The American Institute of Nutrition (AIN) diets are purified ingredient diets that have been developed to be comparable with standard chow diets. Thus both purified diet and natural ingredient chow diets are commonly used in laboratory experiments. However, these diets may not be used interchangeably due to recent suggestions that the chow diets contain natural endocrine disruptors known as phytoestrogens (5). Dietary phytoestrogens are plant-derived compounds that are structurally and/or functionally similar to estrogens. Phytoestrogens act in a biphasic manner, indicated by estrogenic activity in the presence of low endogenous estrogen and antiestrogenic activity in the presence of high endogenous estrogen (5). Soy, one of many foods rich in phytoestrogens and a common ingredient in laboratory animal chow diets, has recently been identified as...
a confounding element in reproductive and endocrine studies (5, 19). Therefore, chow diets may be inappropriate for studies examining reproductive endpoints.

This study compared use of purified vs. chow as standard diets due to concerns about the presence of dietary phytoestrogens in the natural ingredient chow diets exerting reproductive effects. It is critical to understand the role of diet on female fertility. Similarly, the reproductive implication of exposing females to the unique environment of space, i.e., hypogravity, was anesthetized with isoflurane, bled by cardiac puncture, and then decapitated. Blood was collected to determine the levels of luteinizing hormone (LH), follicle-stimulating hormone (FSH), estradiol, progesterone, and corticosterone (CORT). Reproductive and major organs were excised, trimmed, bloated, and weighed.

**Estrous cycle evaluation.** Daily vaginal smears were performed (4). Briefly, the tip of a saline-dampened cotton swab was gently introduced into the vagina, with care being taken not to insert to a depth of >1.0 cm; otherwise, pseudopregnancy might have been stimulated in rats. The smear was transferred onto a microscope slide and examined under a light microscope (×20) to determine the stage of the estrous cycle. The stages of the estrous cycle were determined using the method of Everett (10). The estrous cycle stages are 1) proestrus (mainly epithelial cells), 2) estrus (mainly cornified cells), 3) metestrus (cornified and leukocytes), and 4) diestrus (mainly leukocytes) present. A normal estrous cycle in rats was defined as 4–5 days.

**Urinary CORT measurement.** The NASA-designed HLS cage also functions as a metabolic cage (14). Daily 24-h urine samples were collected to account for the diurnal variation in CORT levels. Five-day, 24-h urine samples were collected to take into account the hormonal fluctuations occurring during the estrous cycle. Urine volumes and samples were collected at three time points: baseline (i.e., preexperimental), at the initiation of the experiment, and at the end of the experimental period. Decalyne oil (1 ml) was added to the urine tubes before collection to prevent evaporation. Collected 24-h urine samples were centrifuged at 1,500 g for 10 min at 4°C. After centrifugation, urine samples were aliquoted into fresh tubes and kept frozen at −20°C until assayed for CORT and creatinine. Urinary concentration of creatinine was measured using an automated analytical system (COBAS; Roche Diagnostic Systems, Somerville, NJ).

**Urinary CORT levels** were determined according to Ortiz et al. (26). Briefly, to determine urinary CORT levels, an aliquot of 250 μl was extracted using 1:1 dichloromethane. After extraction, urine samples were reconstituted 1:20 using steroid diluent and urinary CORT level determined by commercially available rat CORT ImmunoChem double-antibody radioimmunoassay (RIA) kit (ICN Biomedicals, Costa Mesa, CA). Radiosotope counting was done using a Cobra II Auto-Gamma counter (Packard BioScience, Downers Grove, IL). All samples were measured in duplicate. The intra- and interassay coefficients of variation for urinary CORT were 3.6 and 4.3%, respectively.

**Plasma hormone measurements.** Blood samples were obtained from the rats at dissection. Animals were anesthetized with isoflurane and then bled by cardiac puncture. Blood was collected in tubes containing heparin and kept on ice. Collected blood samples were centrifuged at 1,500 g for 10 min at 4°C to separate plasma. Plasma samples were collected and stored at −80°C until assayed for pituitary, reproductive, and stress hormones. Commercially available Coat-a-Count (Diagnostic Products, Los Angeles, CA) RIA kits were used to measure plasma concentrations of estradiol and progesterone. Plasma LH and FSH were determined by double-antibody RIA kit (Amersham, Arlington Heights, IL). ImmunChem double-antibody RIA kits (ICN Biomedicals) were used to determine plasma CORT levels. The intra- and interassay coefficients of variation were 6.2 and 7.5% for estradiol, 4.3 and 6.3% for progesterone, 3.7 and 5.4% for LH, 6.7 and 7.3% for FSH, and 5.0 and 5.3% for CORT, respectively.

**Pituitary hormone measurements.** Pituitary glands were dissected, and the anterior pituitary was separated from the neural lobe, weighed, and frozen at −80°C until assayed. To determine pituitary LH and FSH levels, each anterior pituitary was homogenized in 1 ml of ice-cold phosphate buffer (0.025 M, pH 7.5, containing 0.1% wt/vol sodium azide). The homogenate was centrifuged at 1,500 g for 10 min at 4°C. The supernatant obtained following centrifugation was diluted 1:320 in ice-cold phosphate buffer. Pituitary LH and FSH were determined by double-antibody RIA kit (Amersham). The intra- and

**Materials and Methods.** All procedures used in this study conformed to the National Research Council guide for the use and care of laboratory animals (24). The animal protocol for this study was approved by the Institutional Animal Care and Use Committee at the National Aeronautics and Space Administration (NASA) Ames Research Center.

**Animals and diets.** Forty-two 50-day-old virgin female Sprague-Dawley-derived albino rats were received from Simonsen Laboratories (Gilroy, CA). Upon receipt, animals were individually housed in NASA-designed HLS/metabolic cages (14) in a room maintained at 22 ± 2°C with a 12:12-h light-dark cycle (6:00 AM lights on/6:00 PM lights off). Upon receipt, rats were randomly assigned to be fed either purified AIN-93G or chow diet and subjected to HLS. Altered estrous cycles and pituitary, reproductive, and stress hormone levels were examined as indicators of compromised reproduction.
ranging in weight from 160 to 163 g were randomly assigned

Results are expressed as means ± SE. n = 9–11 rats/group. Different letters indicate significant differences at P < 0.05 by 1-way ANOVA followed by Tukey's test.

interassay coefficients of variation were 6.2 and 7.5% for pituitary LH and 3.0 and 4.1% for pituitary FSH, respectively.

Organ weights. At dissection, organs were excised, trimmed, blotted, and weighed. The uterus, ovaries, and anterior pituitary were weighed as reproductive indexes. The adrenals were weighed as an indicator of chronic stress. The major organs i.e., brain, liver, and kidneys, were also weighed.

Statistical analysis. Urinary CORT was calculated as hormone level times 24-h urine volume divided by creatinine excretion rate. All statistical analyses were done using StatView (Abacus Concepts, Berkeley, CA). One-way ANOVA was used to determine differences between treatment groups. Two-way ANOVA was used to determine the differences due to diet, HLS treatment or diet times HLS treatment. Post hoc multiple comparison tests were performed using Tukey's test. Differences were considered significant at P < 0.05. Results are expressed as means ± SE.

RESULTS

Body mass and food consumption. Female rats aged 50 days ranging in weight from 160 to 163 g were randomly assigned (n = 20–21 rats/group) to dietary treatments so that the initial body mass of the rat group fed the purified diet (AIN-93G) did not differ from the rat group fed the chow diet.

During the 7-day acclimation, body mass gain and food consumption were similar between the AIN-93G and chow-fed rats. During the 20-day preexperimental period, body mass gains were higher (P = 0.01) in rats receiving AIN-93G (40.4 ± 2.3 g) compared with those receiving chow (30.4 ± 3.2 g). Yet food consumption was lower (P = 0.004) in the AIN-93G (15.7 ± 0.2 g/day) than in chow-fed rats (16.7 ± 0.3 g/day). At the end of the 20-day preexperimental period, rats fed different diets were assigned (n = 9–11 rats/group) to be subjected to HLS or kept as AMB controls. Resulting treatment groups were AMB/AIN-93G, AMB/Chow, HLS/AIN-93G, and HLS/Chow rats. At the start of 38-day HLS, the groups were assigned so that initial body mass did not differ among AMB/AIN-93G (217.5 ± 3.5 g), AMB/Chow (215.2 ± 4.6 g), HLS/AIN-93G (224.9 ± 3.6 g), and HLS/Chow (214.4 ± 5.4 g) rats.

During the 38-day experimental period, the body mass of AIN-93G-fed rats was consistently higher than that of chow-fed animals (Fig. 1) in a given treatment group, HLS or AMB. This was particularly notable in animals subjected to HLS despite HLS/AIN-93G rats having significantly lower food consumption (13.9 ± 0.4 g/day) compared with HLS/Chow rats (15.8 ± 0.4 g). A two-way ANOVA indicated a diet effect (P = 0.01) on body mass. However, there was no difference in final body mass among AMB/AIN-93G (254.7 ± 6.4 g) and AMB/Chow (242.0 ± 6.8 g), HLS/AIN-93G (251.2 ± 7.3 g), and HLS/Chow (238.1 ± 6.1 g) rats.

Estrous cycles. After 7-day acclimation, estrous cycles were determined daily for a 20-day preexperimental period. In the AIN-93G-fed rats, average estrous cycle length (4.7 ± 0.4 days) was not significantly different from chow-fed animals (4.6 ± 0.4 days). However, one rat in the chow-fed group was acyclic, indicated by persistent estrus (i.e., cornified cells in vaginal cytology) for 20 days, and was excluded from the experiment. Only rats that were determined to be cycling normally (4–5 days) were used in the 38-day experiment.

HLS increased mean average cycle length compared with AMB control rats (Fig. 2). A two-way ANOVA indicated an HLS treatment effect (P = 0.002) but no diet or diet times HLS

Fig. 1. Effect of diet and/or exposure to hindlimb suspension (HLS) on body mass during the 38-day experimental period. AMB/AIN-93G, ambulatory (AMB) rats fed American Institute of Nutrition (AIN)-93G diet; AMB/Chow, AMB rats fed chow diet; HLS/AIN-93G, HLS rats fed AIN-93G diet; HLS/Chow, HLS rats fed chow diet. Values are means ± SE; n = 9–11 rats/group. Different letters indicate significant differences at P < 0.05 by 1-way ANOVA followed by Tukey's test.

Fig. 2. Effect of diet and/or exposure to HLS on average estrous cycle lengths. Values are means ± SE; n = 9–11 rats/group. Different letters indicate significant differences at P < 0.05 by 1-way ANOVA followed by Tukey’s test.
treatment effect on estrous cycle length. Figure 3 shows the percentage of rats with normal (4–5 days) estrous cycles/cycles during the 38-day HLS. During initial 5-day HLS (cycle 1), the percentage of normally cycling rats decreased compared with AMB rats. However, the estrous cycle following adaptation to HLS (cycle 2) showed that rats returned to normal cycling. By cycles 3 and 4 (days 13–20 HLS), HLS rats showed a greater percentage of animals with lengthened estrus cycles compared with AMB rats.

Rats subjected to HLS had lengthened cycles due to prolonged diestrus, i.e., total number of days in diestrus during the 38-day experimental period (Fig. 4). HLS rats also demonstrated reduced time spent in estrus; however, this occurred only in rats fed AIN-93G and not in rats fed the chow diet. A two-way ANOVA on time spent in diestrus or estrus indicated a diet times HLS treatment effect \( P < 0.05 \). Diet, HLS treatment, and the combination of diet times HLS treatment had no significant effect on time spent in proestrus or metestrus.

**Urinary CORT.** Urinary CORT was collected at baseline (preexperimental), during initial HLS, and at the end of the experiment. Figure 5 shows that baseline urinary CORT was significantly higher in chow-fed rats compared with AIN-93G-fed animals, indicating a diet effect. Initial HLS resulted in elevated urinary CORT levels compared with AMB controls, with no significant differences between rats fed either the chow or AIN-93G diet. A two-way ANOVA indicated an HLS treatment effect \( P < 0.001 \) but no diet or treatment times diet effect on urinary CORT. After 4 to 5 days of exposure to HLS, urinary CORT levels returned to baseline levels (Fig. 6). At the end of the experimental period, urinary CORT levels in HLS rats were similar to baseline levels. Final CORT levels were higher in HLS/Chow rats vs. AIN-93G-fed animals (Fig. 5).

**Plasma and pituitary hormones.** Plasma estradiol levels were significantly lower in HLS/AIN-93G rats compared with AMB/AIN-93G rats (Table 1). There was no significant effect of diet, HLS treatment, or diet times HLS treatment on plasma progesterone or plasma and pituitary LH or FSH levels (Table 1). Plasma CORT levels were significantly higher in HLS/Chow than in AMB/AIN-93G rats (Table 1). There was a trend of increased plasma CORT levels in chow- vs. AIN-93G-fed rats and in HLS vs. AMB rats.

**Organ weights.** Absolute and relative weights (mg/100 g body mass) of organs are shown in Table 2. In this study, there was no effect on absolute or relative adrenal weights, an indicator of chronic stress. There were no differences in ovarian or uterine weights among the different treatment groups; however, absolute weight of the anterior pituitary was lower \( P = 0.04 \) in HLS/AIN-93G rats.

There were no significant effects on absolute or relative posterior pituitary weight among the different treatment groups. However, relative kidney weight was significantly higher in HLS rats compared with AMB/AIN-93G rats. Of the other major organs, absolute liver weight was lower in HLS/AIN-93G than in AMB/Chow rats. Corrected for body weight differences, liver weight was lower \( P < 0.05 \) in the AIN-93G-fed rats compared with the chow-fed animals. There was no significant effect on absolute or relative brain or heart weights among the different treatment groups.

**DISCUSSION**

The HLS model used to simulate the major physiological changes associated with hypogravity produced lengthened estrous cycles. Lengthened estrous cycles in rat have important implications on reproduction because it reduces the cumulative number of cycles. This potentially compromises fertility by reducing the number of potential matings that may occur over the animal’s life span. Also, cycle irregularity is associated with anovulation, which decreases the ability to achieve a fertile mating.

Lengthening of the estrous cycle in HLS rats was caused by prolonged diestrus (Fig. 4). Similarly, exposure to hypergravity by means of centrifugation (1.39–1.65 g) lengthened estrous cycles due to prolonged diestrus (20). Despite prolonged diestrus in HLS rats, there was no significant effect on plasma...
progesterone. In contrast, Megory et al. (20) reported elevated progesterone during prolonged diestrus in rats exposed to centrifugation, i.e., hypergravity. Failure to detect elevated plasma progesterone in this study may have resulted because animals were killed in estrus rather than diestrus, when progesterone levels are elevated.

During diestrus, estradiol levels are low, suggesting that the prolonged diestrus observed in animals exposed to HLS was caused by hypoestrogenism. In support, measurement of plasma estradiol indicated reduced endogenous estrogen in HLS/AIN-93G rats (Table 1). To our knowledge, we are the first to measure estrous cycling and sex steroid levels in HLS female rats. In male rats, exposure to HLS resulted in reduced T (26, 38). Similarly, decreased T in male rats has been consistently observed following space flight (1, 38). Space flight studies measuring reproductive hormone levels in females are nonexistent. However, similar regulation of the hypothalamic-pituitary-gonad (HPG) axis between the sexes suggests that estrogen levels will be suppressed in females.

Diet was longer in HLS rats fed AIN-93G than in those fed the chow diet. Also, the HLS/AIN-93G but not the HLS/Chow rats had reduced time spent in estrus and decreased plasma estradiol levels. This suggested that reductions in estrogen induced by HLS were attenuated in chow-fed rats. Soy meal, a major ingredient in the chow (TD 8728C) diet, contains an estimated 496-μg phytoestrogen/g diet (personal communication, Dr. Charles Benton, Harlan Teklad, WI), a level present in commercial chow diets reported to exert reproductive effects (5). Phytoestrogens act in a biphasic manner indicated by estrogenic activity in the presence of low endogenous estrogen and antiestrogenic activity in the presence of high endogenous estrogen. Thus the hypoestrogenism produced by HLS may have been counteracted in part in the chow-fed rats by the presence of dietary phytoestrogens acting as an exogenous source of estrogen. The results underscore the importance of considering the type of diet when planning or comparing experiments measuring endocrine-sensitive endpoints.

Diet influenced HLS effects on the estrous cycles. However, the mechanism of action whereby HLS affects estrous cycling remains to be determined. The estrous cycle is regulated by the HPG axis. Thus endocrine changes may be exerted at the level of the hypothalamus, pituitary, or gonads. Lower absolute anterior pituitary weight in HLS/AIN-93G rats suggested that disrupted regulation of estrous cycles occurred at the pituitary or hypothalamus. In postpartum space flight rats, pituitary LH was reduced, but there was no effect on plasma LH (6). Plasma FSH was elevated, but there was no effect on pituitary FSH (6). In our study, there was no significant difference in either plasma or pituitary LH and FSH levels between the treatment groups (Table 1). We performed a single blood measurement at necropsy rather than sequential blood sampling of LH and FSH to minimize stress to animals.

Initial HLS produces an acute stress response indicated by elevated 24-h urinary CORT during the first 4 days of HLS compared with AMB controls (Fig. 6). Similarly, Ortiz et al. (26) reported a rise in urinary CORT levels in male rats due to the stress produced by initial HLS. Regulation of the HPG axis is closely linked to the hypothalamic-pituitary-adrenal (HPA) axis. Various types of stressors have been shown to desynchronize estrous cycling in the rat (27). Studies of the underlying mechanism of stress-induced reproductive dysfunction indicated that effects are mediated via alterations in the reproductive endocrine system (28). In the present study, HLS induced a decline in plasma estrogen levels but no effect on LH and FSH (Table 1). Charpenet et al. (8) reported that chronic, intermittent immobilization stress of male rats resulted in reduced plasma T despite no apparent effect on plasma LH levels.

If stress-associated physiological change is the mechanism disrupting estrous cycling in HLS, then endogenous estrogen level becomes an important factor to examine. This is because estrogen plays a role in enhancing the stress response in females based on increased HPA response to stress when ovariectomized rats are treated with estradiol (7). In female rats
subjected to restraint stress on the day of proestrus, when estrogen peaks, there is an almost complete suppression of the preovulatory LH surge (30). Viau and Meaney (39) reported that female rats displayed enhanced plasma adrenocorticotropin hormone (ACTH) and CORT levels to a stressor in proestrus, when estrogen levels are high, compared with diestrus, when estrogen levels are low. We did not control for the phase of the cycle at initiation of HLS of rats, since stress produced by initial HLS lasts 4 days, the length of an estrous cycle.

We investigated the potential of dietary estrogens present in chow diet to affect the stress response to initial HLS. Given their widespread occurrence in plants, the environmental estrogens most likely to be encountered are the phytoestrogens

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**Fig. 5.** Effect of diet and/or exposure to HLS on 5-day urinary corticosterone/creatinine. Values are means ± SE; n = 9–11 rats/group. Different letters indicate significant differences at P < 0.05 by 1-way ANOVA followed by Tukey’s test.

**Fig. 6.** Effect of diet and/or exposure to HLS on 24-h urinary corticosterone/creatinine collected during the initial 5 days of HLS. Values are means ± SE; n = 9–11 rats/group. Different letters indicate significant differences at P < 0.05 by 1-way ANOVA followed by Tukey’s test.
DIET AND HINDLIMB SUSPENSION AFFECT ESTROUS CYCLING

Effect of diet and/or exposure to hindlimb suspension on organ weights

Table 1.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Estradiol, pg/ml</th>
<th>Progestosterone, ng/ml</th>
<th>Corticosterone, ng/ml</th>
<th>LH, ng/ml</th>
<th>FSH, ng/ml</th>
<th>Pituitary LH, ng/gland</th>
<th>Pituitary FSH, ng/gland</th>
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</thead>
<tbody>
<tr>
<td>AMB/AIN-93G</td>
<td>11</td>
<td>18.7±2.8†</td>
<td>10.5±2.8</td>
<td>273.5±53.1†</td>
<td>1.31±0.03</td>
<td>2.10±0.23</td>
<td>432.0±75.7</td>
<td>128.8±10.2</td>
</tr>
<tr>
<td>AMB/Chow</td>
<td>10</td>
<td>14.5±3.5†</td>
<td>10.2±5.9</td>
<td>344.0±52.0†</td>
<td>1.34±0.02</td>
<td>1.69±0.07</td>
<td>383.3±31.7</td>
<td>122.9±13.0</td>
</tr>
<tr>
<td>HLS/AIN-93G</td>
<td>7</td>
<td>7.6±1.5†</td>
<td>14.6±7.0</td>
<td>397.1±90.6†</td>
<td>1.29±0.02</td>
<td>1.86±0.02</td>
<td>521.4±68.5</td>
<td>155.3±7.5</td>
</tr>
<tr>
<td>HLS/Chow</td>
<td>9</td>
<td>12.8±1.3†</td>
<td>9.8±4.5</td>
<td>487.2±34.4†</td>
<td>1.29±0.01</td>
<td>1.87±0.16</td>
<td>435.8±49.9</td>
<td>146.2±10.5</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 7–11 rats/group. AMB/AIN-93G, ambulatory (AMB) rats fed American Institute of Nutrition (AIN)-93G diet; AMB/Chow, AMB rats fed chow diet; HLS/AIN-93G, hindlimb- suspended (HLS) rats fed AIN-93G diet; HLS/Chow, HLS rats fed chow diet. Different symbols in the same column indicate significant differences at P < 0.05 by 1-way ANOVA followed by Tukey’s test.

(12). Recently, elevated plasma ACTH and hippocampal glucocorticoid receptor levels following acute immobilization stress was reported in male rats given lifelong consumption of a high-phytoestrogen diet (600 µg phytoestrogen/g) (18). Hartley et al. (15) reported that adult male rats fed 150 µg phytoestrogen/g for 14 days had elevated plasma CORT and vasopressin levels when exposed to elevated plus maze and social interaction. No studies have been performed on females despite the higher stress reactivity in females compared with males (7).

Baseline urinary CORT levels were significantly higher in rats fed chow compared with those fed the phytoestrogen-free AIN-93G diet, suggesting that dietary sources of estrogens are capable of enhancing the stress response in intact, cycling rats. Exposure to HLS resulted in elevated urinary CORT levels. However, a two-way ANOVA indicated a significant HLS treatment effect (P < 0.001) but no effect of either diet or HLS treatment times diet effect on urinary CORT levels. The estrogenicity of the chow diet used in this study did not appear to be of sufficient dose to elevate the stress response to initial HLS. Still, caution must be used when considering the experimental diet, because to our knowledge no studies have systematically investigated whether dietary estrogen can enhance stress response and whether this, in turn, affects female reproductive health.

Interestingly, irregular cycling occurred at 15–20 days (~3–4 cycles) of HLS exposure, after adaptation to HLS (Fig. 3). Adaptation to the acute stress produced by HLS was indicated by the return of urinary CORT to baseline levels 4–5 days after initial HLS (Fig. 6) and by the absence of significant differences in body mass and adrenal weights, indicators of chronic stress, between HLS and AMB rats (Table 2). However, measurement of urinary and plasma CORT at the end of the 38-day experimental period indicated elevated stress hormones in HLS/Chow rats compared with AMB/AIN-93G rats. The chow diet may contain epigenetic factors, such as phytoestrogen, capable of inducing alterations after initial stress exposure that results in increased sensitivity to later stress. On dissection day, inadvertent exposure to stress may have been introduced by such subtle actions as transporting animals to the necropsy room.

Unfortunately, on the basis of this study it was not possible to determine unequivocally whether dietary phytoestrogens provided an exogenous estrogen source that affected CORT levels and estrous cycling due to caloric and ingredient differences between purified vs. chow diets. Although purified diet and chow are used interchangeably as standard diets, it is important to consider the differences between them, particularly in studies measuring reproductive end points.

Table 2.

<table>
<thead>
<tr>
<th>Measurements</th>
<th>AMB/AIN-93G (n = 11)</th>
<th>AMB/Chow (n = 10)</th>
<th>HLS/AIN-93G (n = 7)</th>
<th>HLS/Chow (n = 9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body wt, g</td>
<td>254.7±6.4</td>
<td>242.0±6.8</td>
<td>251.4±7.3</td>
<td>238.1±6.1</td>
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<tr>
<td>Absolute uterine wt, mg</td>
<td>493.5±21.7</td>
<td>504.9±30.1</td>
<td>450.2±64.0</td>
<td>522.0±45.6</td>
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<tr>
<td>Relative uterine wt, mg/g body wt</td>
<td>194.4±8.8</td>
<td>210.4±13.0</td>
<td>191.9±29.2</td>
<td>224.1±19.6</td>
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<tr>
<td>Absolute ovarian wt, mg</td>
<td>125.8±8.9</td>
<td>113.6±7.5</td>
<td>136.2±27.4</td>
<td>122.5±4.5</td>
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<tr>
<td>Relative ovarian wt, mg/g body wt</td>
<td>49.0±2.0</td>
<td>46.9±2.9</td>
<td>56.2±10.2</td>
<td>52.6±2.1</td>
</tr>
<tr>
<td>Absolute anterior pituitary wt, mg</td>
<td>9.3±0.5†</td>
<td>7.9±0.4†</td>
<td>7.6±0.3*</td>
<td>8.7±0.7†</td>
</tr>
<tr>
<td>Relative anterior pituitary wt, mg/g body wt</td>
<td>3.7±0.3</td>
<td>3.6±0.2</td>
<td>3.2±0.2</td>
<td>3.7±0.2</td>
</tr>
<tr>
<td>Absolute posterior pituitary wt, mg</td>
<td>4.6±1.3</td>
<td>3.8±1.1</td>
<td>2.3±0.9</td>
<td>4.4±1.3</td>
</tr>
<tr>
<td>Relative posterior pituitary wt, mg/g body wt</td>
<td>1.8±0.5</td>
<td>1.6±0.5</td>
<td>1.0±0.4</td>
<td>1.9±0.5</td>
</tr>
<tr>
<td>Absolute adrenal wt, mg</td>
<td>61.1±1.9</td>
<td>57.5±1.7</td>
<td>61.0±2.0</td>
<td>61.9±2.8</td>
</tr>
<tr>
<td>Relative adrenal wt, mg/g body wt</td>
<td>24.1±0.9</td>
<td>23.8±0.8</td>
<td>25.8±1.5</td>
<td>26.5±1.1</td>
</tr>
<tr>
<td>Absolute brain wt, mg</td>
<td>1,547.6±78.6</td>
<td>1,647.0±163.3</td>
<td>1,537.7±125.7</td>
<td>1,557.7±88.7</td>
</tr>
<tr>
<td>Relative brain wt, mg/g body wt</td>
<td>611.5±34.7</td>
<td>684.7±17.2</td>
<td>640.9±43.6</td>
<td>671.2±43.6</td>
</tr>
<tr>
<td>Absolute kidney wt, mg</td>
<td>1,608.7±37.2</td>
<td>1,628.9±45.9</td>
<td>1,621.5±40.2</td>
<td>1,664.2±56.0</td>
</tr>
<tr>
<td>Relative kidney wt, mg/g body wt</td>
<td>633.3±12.9†</td>
<td>674.3±12.7†</td>
<td>6.83±22.2†</td>
<td>712.7±18.2†</td>
</tr>
<tr>
<td>Absolute heart wt, mg</td>
<td>837.4±27.4</td>
<td>795.6±20.8</td>
<td>856.7±25.9</td>
<td>784.5±21.5</td>
</tr>
<tr>
<td>Relative heart wt, mg/g body wt</td>
<td>328.9±6.8</td>
<td>329.5±7.1</td>
<td>360.7±10.9</td>
<td>305.5±35.1</td>
</tr>
<tr>
<td>Absolute liver wt, mg</td>
<td>7,449.5±219.0††</td>
<td>7,917.8±341.0††</td>
<td>7,027.6±230.7*</td>
<td>7,854.6±320.2*†</td>
</tr>
<tr>
<td>Relative liver wt, mg/g body wt</td>
<td>2,928.1±59.6*</td>
<td>3,263.5±71.1††</td>
<td>2,948.9±41.0*</td>
<td>3,362.7±115.3†</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 7–11 rats/group. Different symbols in the same column indicate significant differences at P < 0.05 by 1-way ANOVA followed by Tukey’s test.

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In our study, rats fed the purified (AIN-93G) diet had lower food intakes than chow-fed animals. Mild food restriction has been reported to influence estrous cycling in rats (34). However, disruption of estrous cycling due to mild food deprivation was associated with body weight loss. In contrast, in this study, rats fed the purified diet consumed less than chow-fed animals but had a higher body mass. This was because the caloric density of the AIN-93G diet (4.03 kcal/g) was greater than that of the Chow diet (3.10 kcal/g). Anastasia et al. (2) also observed higher body weights with lower food intakes in rats fed purified vs. chow diet.

Because of the greater caloric density, rats fed the AIN-93G diet may have been overfed. Overfeeding has been reported to lengthen estrous cycles due to prolonged diestrus phase (11). However, this occurred when obesity developed, which was not the case in this 38-day study. Further evidence that caloric differences and body mass did not affect estrous cycling in this study was the observation of the absence of disrupted estrous cycles in the AMB control rats fed purified or chow diet and lengthened estrous cycles in HLS rats fed either diet (Fig. 2). Thus HLS rather than diet appeared to have been the factor influencing estrous cycle length.

Nelson and Felicio (25) reported that feeding mice diets that were nearly isocaloric but differing in nutrient composition resulted in differences in estrous cycle length and acyclicity. Purified diets considered phytoestrogen-free have been reported to produce estrogenic activity (36). Mice fed estradiol in the purified AIN-76A diet showed a greater estrogenic response compared with mice given estradiol in the NIH-07 chow diet (13). Thigpen et al. (35) suggested that high sucrose, dextrose, and cornstarch were responsible for producing estrogenic effects. Compared with the AIN-76A diet, the AIN-93G diet used in our study was higher in cornstarch but lower in sucrose and dextrose.

Another dietary effect on endocrine end points is the protein source. The protein source in the AIN-93G diet is casein, whereas the source in the chow (TD 8728C) is soybean protein. Dietary casein has been reported to elevate circulating cholesterol levels in rats compared with soybean protein (16). Altered cholesterol metabolism may have produced the changes in plasma estrogen observed. However, Badger et al. (3) reported that casein- vs. soy-fed male rats did not differ in hepatic metabolism of reproductive hormones. Thus the phytoestrogens in chow seemed to be the most plausible dietary component responsible for the observed endocrine effects. However, to determine this, a known concentration of the phytoestrogen is required to be mixed into a phytoestrogen-free diet such as AIN-93G, while calories and ingredients of treatment diets are kept the same.

In conclusion, HLS induced endocrine changes that altered ensuing estrous cycles, and this, in turn, may potentially affect fertility. An interesting finding was the ability of diet to attenuate hypoestrogenism by influencing endogenous estrogen levels. Traditionally, researchers have relied on standard laboratory chow. Similarly, ground-based studies simulating space flight employ standard rodent chow, yet recent diet formulations for space flight rodents resemble purified diets (37). Although this may provide acceptable baselines for various studies, this may not be the case when reproductive and endocrine end points are examined. Presently, the mechanism whereby HLS affects estrous cyclicity is unclear, and more research is required. A better understanding of environmental influences on ovulatory cycles will contribute to reproductive success in space and improve fertility and female reproductive health on Earth.

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GRANTS

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REFERENCES

Diet and Hindlimb Suspension Affect Estrous Cycling